



## Research Article

**EVALUATION OF TOPICAL ANTI-INFLAMMATORY EFFECT OF AZADIRACHTA INDICA LEAF EXTRACT**

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**Abstract:** This study aimed to evaluate the possible anti-inflammatory effects of separate ointment formulations of three extracts of *Azadirachta indica* (*A. indica*) for topical administration. It also compared the prepared formulation with a standard ointment in the market (Volini gel) by using the carrageenan induced hind-paw model and skin punch biopsy method in experimental rats by topical application. The petroleum ether, chloroform and alcohol extracts of the leaves of *A. indica* were prepared into an ointment, which contained 5% of each extract separately in a cream base. Among the prepared formulations of the plant, the petroleum ether extract of *A. indica* showed the maximum edema inhibition, which was similar to that of Diclofenac gel. The inhibition of the inflammation in both the models suggests that *A. indica* may be used for managing the inflammatory conditions.

**Key words:** Anti-inflammatory activity, Carrageenan, *Azadirachta indica*, Topical application, Turpentine oil

**INTRODUCTION**

Natural products are significant sources of synthetic and traditional herbal medicines and are still the primary health care system<sup>1</sup>. Many of today's modern drugs like opium, aspirin, digitalis, and quinine have their origin from plants<sup>2</sup>. Herbal practitioners throughout the world have described the therapeutic efficacies of many indigenous plants for several disorders<sup>3</sup>. Moreover plants are the sources of many life-sustaining metabolites, extensive research is still being done on the use of plants in healing various diseases.

*Azadirachta indica* A. Juss (commonly called neem) is a plant species belonging to the family Meliaceae. It is an attractive evergreen tree, well known in India and its neighboring countries for more than 2000 years, as one of the most versatile medicinal plants with a wide spectrum of biological activities. It is estimated that about 75% of Ayurvedic formulations contain some parts of the *A. indica* tree in one form or other. The importance of this tree has been recognized by the US National Academy of Sciences through its report entitled 'Neem: A tree for solving global problems', published in 1992<sup>4</sup>. Extensive chemical investigations were carried out in the middle of the twentieth century for isolation of chemical compounds from *A. indica*. Nimbin, the first bitter compound of *A. indica* was extracted from its oil<sup>5</sup>. Nimbidin, is another major crude active

principle extracted from the oil of seed kernels of *A. indica*, which has shown several biological activities. Later on, more than 135 compounds have been isolated from various parts of *A. indica*. Moreover, several reviews have also been published on the chemistry and structural diversity of these compounds<sup>6</sup>. Singh<sup>7</sup> in his book "Neem, a treatise" reported that *A. indica* leaf mainly contains quercetin (flavonoid), nimbosterol ( $\beta$ - sitosterol), and a number of liminoids (nimbin and its derivatives). The principal constituents of *A. indica* leaf include protein (7.1%), carbohydrates (22.9%), minerals, calcium, phosphorus, vitamin C, carotene etc. It also contains glutamic acid, tyrosine, aspartic acid, alanine, proline, glutamine and cystine like amino acids, and several fatty acids (dodecanoic, tetradecanoic, elcosanic, etc.).

Modern scientific investigations have elucidated the biological activity and usage of crude extracts and their fractions from leaf, bark, root, seed, and oil of *A. indica* as traditional medicine for treatment of various ailments. Tidjani et al<sup>8</sup> reported that the chloroform extract of stem bark is effective against carrageenan induced paw oedema in rat and mouse ear inflammation. Okpanyi and Ezeukwu demonstrated the antipyretic effects of methanol extract of *A. indica* leaves in male rabbits<sup>9</sup>. Vohora and Dandiya reported that the plants possess analgesic activity mediated through opioid receptors

in laboratory animals<sup>10</sup>. According to Subapriya & Nagini Neem leaf exhibits a wide range of pharmacological activities viz., anti-inflammatory, antihyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic, anticarcinogenic and immunomodulatory<sup>11</sup>. Bark, leaf, root, flower and fruit together have been used to cure blood morbidity, biliary afflictions, itching, skin ulcers, burning sensations and phthisis<sup>6</sup>. However, the anti-inflammatory activity on topical application has not yet been elucidated. Hence we report the anti-inflammatory effect of three separate formulations made from extracts of *A. indica* leaves in petroleum ether, chloroform and ethanol in an ointment-form for topical administration. The study also aimed to compare the effects of the prepared ointments with a standard gel in the market, Volini gel (1% Diclofenac sodium), by using the inhibition of carrageenan-induced paw edema model and skin Punch method.

## MATERIAL AND METHODS

### Plant collection and preparation of extracts:

Fresh leaves of *A. indica* collected from Nalgonda district of Andhra Pradesh, were first washed to remove dirt and other impurities. They were then air dried in shade, powdered by a mechanical grinder, passed through a 40-mesh sieve and stored in airtight containers for future use. The extracts were prepared by simple maceration technique at room temperature (22-25°C). In this technique, 40 g of leaf powder was soaked separately in 800 ml of each organic solvents - chloroform, petroleum ether, and ethyl alcohol, for 48 hrs. They were stirred intermittently for proper mixing. After 48 hours, the soaked powder was squeezed and the solvents were filtered using Whatman No.1 filter paper. The filtrates were collected and solvents were concentrated at 35°C in petri plates till the entire solvent is evaporated. The murk and the petroleum ether, chloroform and alcohol extracts were weighed to determine the percentage of extracts obtained. The extracts were then stored in the refrigerator at -20°C for future use. The yield was estimated as a percentage of the initial quantity of plant material used (i.e., 40.0 g).

(%) yield = yield in (g) x 100/ 40.0g

### Formulation of topical preparation

The extract obtained was a gummy semi-solid mass (dark green in colour). A 5%w/w cream of each extract was prepared in lipophilic cream base, that contains cetostearyl alcohol, wool alcohols, and

white soft paraffin in the ratio 0.5:6:93.5, respectively. About 5g of the semisolid extract per 100 g of simple ointment base was prepared. Simple ointment base was used in control group (Negative control). Diclofenac sodium (Volini gel) was used as standard for the topical application for standard group.

## Pharmacological assays

### Experimental animals

Wistar male albino rat, weighing 150-200 g, obtained from the Central animal house of Kamineni Institute of Medical Sciences, Nalgonda, Andhra Pradesh were used. They were housed at room temperature (22±3°C) with a 12:12-hrs light and dark cycle. They had free access to food and water, except during the time of experiments. The animals were habituated to laboratory conditions for 48 hrs prior to the experiment to minimize any nonspecific stress. All animal procedures were done in accordance with the approved protocol for use of experimental animals, set by the Standing Committee on Animal Care.

### Grouping of the animals

The experimental animals were first divided into five groups of six animals each. Group I animals, treated with simple ointment (SO) base, served as the control; Group II animals treated with Diclofenac gel (DG), served as standard; Group III animals were treated with Petroleum ether extract (PeE), and served as test I; Group IV animals were treated with Chloroform extract (CE), and served as test II; and Group V animals were treated with alcohol extract (AE), and served as test III.

### Inhibition of carrageenan-induced paw edema

The effect of test drug on carrageenan-induced paw edema was studied in rat paw by the method of Winter *et al*<sup>12</sup>. In order to measure paw volume, the animals were marked with a permanent marker at the ankle of their hind paw to define the area to be monitored. 0.2 g of the prepared ointments of various extracts and the marketed formulation of diclofenac sodium, were applied to the planter surface of the left hind paw by gently rubbing 50 times with the index finger. The ointment base without drug was applied in control group of rats by the same method. After 1 hr, approximately 0.1 ml of 1% (w/v) carrageenan (Sigma Co., USA) suspension (in sterile normal saline) was injected into the sub-plantar surface of the left hind paw. Paw volumes were measured by mercury plethysmograph at regular time intervals at 2, 4 & 6 hrs after the injection. The difference in the

paw volume was calculated by subtracting the baseline reading from that of 2-, 4- and 6- hours' readings and the percentage inhibition in paw volume was calculated using the following formula:

$$\% \text{ inhibition of edema} = 100(1 - V_t/V_c)$$

where,  $V_t$  = mean volume of the paw edema in the drug treated group, and  $V_c$  = mean volume of the paw edema in the untreated control group.

#### Effect of *A.indica* extract on Turpentine oil induced edema:

Thirty albino rats of both sex, weighing between 150-200 g, were used for the study. Animals were randomly divided in five groups of 6 rats each with the help of computer-generated random number. The dorsum of all the animals was shaved two days before the experiment and were adequately disinfected with application of spirit. Various extracts of *A. indica* ointment (0.2g), Diclofenac gel and simple ointment base were rubbed carefully for one minute on the shaved area 30 minutes before the intracutaneous injection of turpentine oil (0.1 ml of 1% solution). After 24 hrs, the application of extract

and injection of turpentine oil were repeated on the same area. The group treated with simple ointment base alone was kept as control. One hour after the second treatment of turpentine oil, the animals were sacrificed and skin punches were obtained with 8 mm diameter cork borer. The skin punches were weighed immediately in an analytical balance and the percent inhibition was calculated by comparing with the control<sup>13</sup>.

#### Statistical analysis

The results obtained are expressed as mean  $\pm$  SEM. The data analysis was done by one-way ANOVA, using the SPSS 18.0 software. The differences between means were compared using Tukey's honest significance test. Probability values of 0.05 or less ( $p < 0.05$ ) were considered statistically significant.

#### RESULTS

The percentage extracts of *A. indica* in various solvents are given in Table 1. It indicates that the maximum percentage extract (35.5%) was obtained in ethanol as the solvent, whereas the minimum extract (14.9 %) was for petroleum ether.

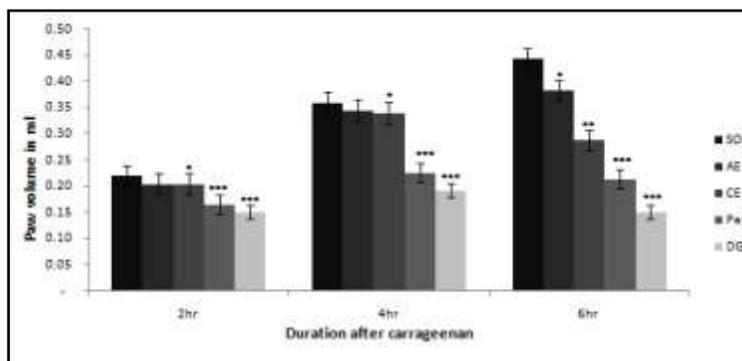
Table.1: The residue weights in the various extracts

S.no	Solvent	Weight in gm			% of extract
		Dry Leaf Powder	Murk	Extract	
1	Chloroform	40	32.6	7.4	22.6%
2	Petroleum ether	40	34.8	5.2	14.9%
3	Ethanol	40	29.5	10.5	35.5%

The results of anti-inflammatory activity after topical application of *A. indica* are presented in Fig 1 and Table 2. Statistical analysis showed that the edema inhibitions of preparations containing extracts are significantly different from that of the control group. There were no significant differences between the formulation containing 1% of Diclofenac sodium (positive control) and the formulation containing 5% of the *A. indica* petroleum ether extract ( $p > 0.001$ ). Diclofenac sodium standard reference drug is

reported to inhibit inflammation by its effect upon plasma exudation associated with carrageenan mediated inflammation<sup>14</sup>.

The paw edema was measured at 2h, 4h and 6h after carrageenan treatment. Animals were challenged with carrageenan and so treated with extracts (Alcohol extract (AE), Chloroform extract (CE), Petroleum ether extract (PeE)). Each bar represents the Mean  $\pm$  SEM for  $n=6$ . \* $P < 0.5$ , \*\* $P < 0.01$  & \*\*\* $P < 0.001$  vs control.

Fig 1. Effect of extracts of *A.indica* and DG topically administered on carrageenan-induced paws edema in rats.

The results obtained from turpentine oil induced skin oedema test are depicted in Table 2. All extracts, except the ethyl alcohol extract, showed significant inhibition of the inflammation compared to control, The values for inhibition of the inflammation was 9.16% for ethyl alcohol, 26.34% for chloroform

extract and 45.23% for petroleum ether extract. As evident, petroleum ether extract was the most effective in inhibiting the inflammation, which was comparable to that of diclofenac gel (49.82%), the drug used as reference.

**Table2: Turpentine oil induced skin edema in rats**

Treatment	Dose	Skin punch (mg) Mean $\pm$ SEM	% Inhibition
Control	Base	87.33 $\pm$ 4.6	—
Diclofenac gel	2%	43.64 $\pm$ 5.5**	49.81
Alcohol Extract	5%	79.33 $\pm$ 6.9	9.16
Chloroform Extract	5%	64.33 $\pm$ 4.3*	26.34
Petroleum EtherExtract	5%	47.83 $\pm$ 5.7**	45.23

Values are mean $\pm$ SEM. \*p<0.05. \*\*p<0.01 vs control

## DISCUSSION

Inflammation has different phases; the early phase is caused by the increase in vascular permeability, the middle by infiltrate of leucocytes and late stages by the granuloma formation. Carrageenan induced paw edema is a widely used method for studying the inflammatory process and for identifying anti-inflammatory agents that could be useful in the treatment of inflammatory disorders. The development of carrageenan-induced oedema is believed to be biphasic. The early phase is attributed to the release of histamine and serotonin and the delayed phase is sustained by the leukotrienes and prostaglandins<sup>15</sup>. Our results clearly show that *A. indica* extract have been able to control the increase in paw edema in the early phase; however it has exhibited significant activity at the 6th hour, which is related to the inhibition of prostaglandins release, which inhibits the late phase of inflammation. Hence, the anti-inflammatory activity of *A. indica* extract

might be due to its action on the later phase of inflammation.

Similar observations were made by a number of researchers with oral administration of *A. indica* leaf extracts. Okpanyi and Ezeukwu studied the anti-inflammatory effects of *A. indica* extracts at a dose of 400 mg/kg body weight, which was comparable to 50 mg/kg acetylsalicylic acid and 4 mg/kg indomethacin<sup>9</sup>. Chattopadhyay et al<sup>16</sup> reported that *A. indica* leaf extracts have significant anti-inflammatory effects against 5-HT and PGEI induced inflammation but not on the inflammation induced by histamine and bradykinin. Pallai and Shanthakumari noticed that Nimbidin, a crude *A. indica* extract at a dose of 80 mg/kg, showed significant anti-inflammatory activity that is almost similar to that of prednisolone at a dose of 10 mg/kg<sup>17</sup>.

Turpentine oil, was used as the phlogistic agent in the second model of inflammation. In this study, skin

punch technique was used to measure the extent of inflammation produced. In the studies conducted by Yamada *et al*<sup>18</sup> on turpentine oil induced subcutaneous inflammation in rats, the histological examination of inflammatory tissue showed the characteristic features of acute inflammation, including migration of neutrophils and edematous subcutaneous tissue on the first day after injury, and chronic inflammation with fibroblasts and vascular proliferation and macrophage infiltration on day 4<sup>19</sup>. The present study showed that topical application of *A. indica* leaf extract is having oedema suppressant activity against turpentine oil induced inflammation. Further studies are needed to substantiate the present preliminary findings. Thus topical application of the *A. indica*-based cream over the inflamed area can provide relief from pain and from inflammation itself.

## CONCLUSIONS

The results presented here support the traditional uses of *A. indica* leaves. From the above results, it can be deduced that petroleum ether extract has shown more significant activity as compared to alcohol and chloroform extract. However further detailed studies is necessary to identify the exact mechanism of action and correlate with the pharmacological activities. Thus supplementing the topical treatment with 5% w/w of petroleum ether *A. indica* leaf extract may be useful for the treatment of local inflammation.

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